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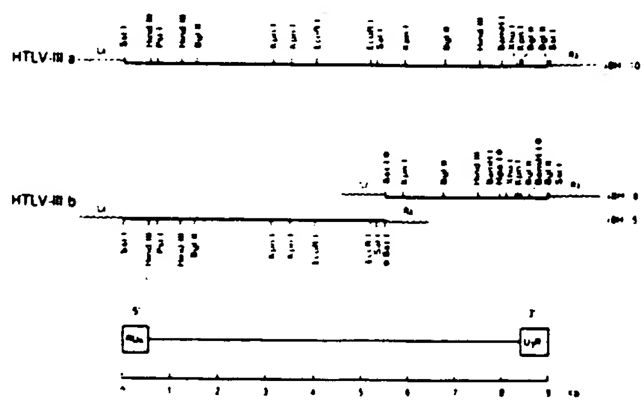
(54) Title: MOLECULAR CLONES OF THE GENOME OF HTLV-III



(57) **Abstract**

Molecular cloning of HTLV-III, the adult leukemia and acquired immune deficiency syndrome (AIDS) virus. Clone BH10 contains a 9.0 Kb viral insert constituting the entire HTLV-III genose. Clones BH8 and BH5 contain viral inserts of 5.5 Kb and 3.5 Kb, respectively. These clones are suitable for the development of diagnostic and therapeutic measures for AIDS, as well as use as probes for the detection of AIDS.

MOLECULAR CLONES OF THE GENOME OF HTLV-III

In related inventions, HTLV-III was detected, isolated and immortalized in an HT cell line. Since evidence now strongly indicates that HTLV-III is related to acquired immune deficiency syndrome (AIDS), the ability to enhance production of the virus and determine the DNA sequences of the virus is critically important to developing a cure or reagent active against AIDS. The present invention takes one such significant step by disclosing the process for molecularly cloning the complete genome of the HTLV-III virus. In short, the molecular cloning of the complete genome of the HTLV-III virus produced by one of these lines designated H9/HTLV-III is disclosed. Two forms of this virus are identified which are highly related but differ in several restriction enzyme cleavage sites. Both variants exist as integrated and unintegrated forms in the infected cell line. The complete genomes of two forms of HTLV-III are molecularly cloned and shown to exist in the long-term infected cell line both as polyclonally integrated provirus and as unintegrated viral DNA. These clones are used as probes to detect viral sequences in cell lines other than H9/HTLV-III, taken from different AIDS patients, and in fresh lymphoid tissues of AIDS patients, providing further evidence that the cloned genomes constitute predominant forms of HTLV-III, the causative agent in AIDS.

Statement of Utility

Previous work with the HTLV family of virus showed three variants. Of these, it was believed that HTLV-III was the causative agent of AIDS. Using the clones produced by this invention, HTLV-III has been shown to be distinctly different than HTLV-I and HTLV-II, whereas HTLV-I and -II share greater homology and thus better identification of AIDS virus in sera.

Description of the Drawings

Figure 1 is a Southern blot analysis of unintegrated DNA of HTLV-III. No viral sequences could be detected in the undigested DNA after 4 hours. However, a major species of viral DNA of approximately 10 Kb length was present in the 10, 15, 24 and 48 hr harvest representing the linear unintegrated form of the virus. A representative Southern blot of the 15 hr harvest digested with several restriction enzymes is shown in this figure. Methods: 8 x 10^8 fresh uninfected H9 cells were infected with concentrated supernatant from cell line H9/HTLV-III containing 4 x 10^{11} particles of HTLV-III. Infected cells were divided into five Roller bottles and harvested after 4, 10, 15, 24 and 48 hrs. Low molecular weight DNA was prepared using the Hirt fractionation procedure and 30 ug of undigested and digested DNA were separated on a 0.8% agarose gel, transferred to nitrocellulose paper and hybridized to a HTLV-III cDNA probe for 24 hr at 37°C in 1 x SSC, 40% formamide and 10% Dextran sulfate. cDNA was synthesized from poly(A) selected RNA prepared from doubly banded HTLV-III virus in the presence of oligo(dT) primers. Filters were washed at 1 x SSC at 65°C.

Figure 2 is a restruction endonuclease map of two closely related HTLV-III variants cloned from unintegrated viral DNA. Three recombinant clones (λBH10,λBH5 and λBH8) were analyzed and their inserts (9 Kb, 5.5 Kb and 3.5 Kb, respectively) were mapped with the indicated enzymes. They represent two variant forms of HTLV-III differing in three enzyme sites which are depicted in bold letters and by an asterisk. As SstI cuts the LTR of the HTLV-III the three clones represent two full length genomes with one LTR. A schematic map of this viral genome is shown at the bottom of the figure, although the total length of the LTR is approximate. Methods: Low molecular weight DNA combined from the 15 and 24 hr harvest was fractionated on a 10-40% sucrose gradient.

Aliquots of the fractions were electrophoresed on a 0.5% agarose gel, transferred to nitrocellulose paper and hybridized to HTLV cDNA under conditions described in Figure 1. Fractions which contained the unintegrated linear HTLV-III genome shown by hybridization were pooled, the DNA was subsequently digested with SstI and ligated to phosphatase treated SstI arms of λgtWesλB. After in vitro packaging, recombinant phages were screened for viral sequences with HTLV-III cDNA.

Figure 3 demonstrates HTLV-III viral sequences in the infected cell line H9/HTLV-III. Both variant forms of HTLV-III were detected as integrated provirus as well as unintegrated viral DNA in the infected cell line. However, no viral sequences were found in uninfected H9 cells, uninfected HT cells nor in normal human thymus (NT). Methods: 10 μg of high molecular weight DNA were digested with restriction enzymes as indicated and hybridized to nick translated phage insert from BH10 under the same conditions as described in Figure 1.

Figure 4 shows a sequence homology of HTLV-III to other members of the HTLV family. A schematic restriction map of HTLV-I, HTLV-Ib and HTLV-II is drawn below indicating the length and the location of the generated fragments in respect to the corresponding genomic regions. LTR, gag, pol, env and pX regions are drawn to scale according to the published nucleotide sequence of HTLV-I. The bands which are most highly conserved as stringency increases correspond to the gag/pol junction region of HTLV-I (1.8 Kb PstI fragment) and HTLV-IIb (3.1 Kb PstI fragment) and to the 3' part of the pol region of HTLV-II (2.1 Kb SmaI/BamHI fragment) which do not overlap assuming the same genomic organization in HTLV-II. Fragments corresponding to pX of HTLV-I (2.1 Kb SstI Pst fragment) and HTLV-Ib (1.4 Kb Pst fragment) are less conserved but still visible at Tm -28°C on the original autoradiogram. Digestion of GaLV generates six fragments, none of which show hybridization under medium or

high stringency. Methods: Subclones of full length genomes of a prototype HTLV-I, HTLV-Ib, HTLV-III and GaLV (Seato strain) were digested with the following enzymes, PstI plus SstI (HTLV-I and HTLV-Ib), BamHI plus SmaI (HTLV-II) and Hind III plus SmaI plus XhoI (GaLV). Four replicate filters were prepared and hybridized for 36 hr under low stringency (8 x SSC, 20% formamide, 10% Dextran sulfate at 37°C) to nick translated insert of λBH10. Filters were then washed in 1 x SSC at different temperatures, 22°C (Tm - 70°C) filter 1, 37°C (Tm - 56°C) filter 2, 50°C (Tm - 42°C) filter 3 and 65°C (Tm - 28°C).

The Invention

The present invention discloses a method for production of molecular clones of HTLV-III from a fraction enriched for the unintegrated provirus in acutely infected cells. Three clones for the HTLV-III genome were produced using recombinant DNA techniques by isolating and characterizing unintegrated viral DNA, cleaving this DNA with the appropriate restriction enzyme, and constructing a phage library capable of being screened by viral cDNA. This process led to the production of three clones: BH10, containing a viral insert of 9.0 Kb corresponding to the complete HTLV-III genome; clone BH8 containing an insert of 5.5 Kb; and clone BH5 containing a viral insert of 3.5 Kb. See Figure 3 for a pictoral representation of the differences between these three clones.

In general, cloning the HTLV-III genome involved isolating unintegrated viral DNA after infection of H9-cells with concentrated HTLV-III virus and cloning this DNA in a lambda phage library to be screened with viral cDNA. The cell line H9/HTLV-III produces large quantities of HTLV-III virus and serves as the principal producer cell line for immunological assays used to detect virus specific antigens and antibodies in AIDS sera. Cultures of H9/HTLV-III cells (infected cells) are

grown and harvested, followed by extraction of low molecular weight DNA from the newly infected cells. This produces unintegrated viral DNA. A cDNA library is formed using HTLV-III cDNA. This cDNA is then used as a probe for assaying the unintegrated viral DNA. Unintegrated linear DNA (provirus DNA) is then obtained, containing the entire HTLV-III genome, i.e., replication competent. This DNA is then digested in plasmid lambda gt Wes · lambda B to form clone lambda BH10. The other clones are produced by digesting provirus DNA that does not contain the entire HTLV-III genome.

Two elements of the above process are recombinant DNA procedures, such as, the DNA library and a cDNA probe. The library is formed by taking the total DNA from H9/HTLV-III cells, cutting the DNA into fragments with a suitable restruction enzyme, hybridizing to the fragments to a radiolabeled cDNA probe, joining the fragments to plasmid vectors, and then introducing the recombinant DNA into a suitable host.

The cDNA probe is an HTLV-III cDNA probe made from double-banded HTLV-III mRNA. A short oligo-dT chain is hybridized to the poly-A tail of the mRNA strand. The oligo-T segment serves as a primer for the action of reverse transcriptase, which uses the mRNA as a template for the synthesis of a complementary DNA strand. The resulting cDNA ends in a hairpin loop. Once the mRNA strand is degraded by treatment with NaOH, the hairpin loop becomes a primer for DNA polymerase I, which completes the paired DNA strand. The loop is then cleaved by S1 nuclease to produce a double-stranded cDNA molecule. Linkers are then added to the double-stranded cDNA by using DNA ligase. After the linkers are cut open with a restriction enzyme and the cDNA is inserted into a suitable plasmid cleaved with the same enzyme, such as pBR322. The result is a cDNA-containing recombinant plasmid.

Statement of Deposit

The cell lines and clones of this invention are on deposit in the American Type Culture Collection in the manner prescribed by the Patent and Trademark Office with regard to permanence of the deposit for the life of the patent and without restriction on public access. The accession numbers are: H9/HTLV-III, CRL 8543; BH10, #40125; BH8, #40127; and BH5, #40126.

Specific Disclosure

Concentrated virus from H9/HTLV-III is used to infect fresh uninfected H9 cells at a multiplicity of 50 viral particles/cell; cultures are harvested after 4, 10, 15, 24 and 48 hours. Extrachromosomal DNA is extracted according to the procedure of Hirt and assayed for its content of unintegrated viral DNA using HTLV-III cDNA as a probe. This cDNA is primed by oligo(dT) and copied from poly(A) containing RNA from virions that had been twice banded on sucrose density gradients. Unintegrated linear viral DNA is first detected after 10 hrs and is also present at the subsequent time points. A Southern blot of the 15 hr harvest is shown in Figure 1. A band of approximately 10 Kb in the undigested DNA represents the linear form of the unintegrated, replication-competent HTLV-III. No closed or nicked circular DNA could be detected in the 10, 15 and 24 hour harvest, but both forms were evident in small amounts at the 48 hr harvest (data not shown). The viral genome was not cut by XbaI, whereas SstI generated three predominant bands of 9 Kb, 5.5 Kb and 3.5 Kb (Figure 1). These bands represent the complete genomes of two forms of HTLV-III, both cut by SstI in the LTR and one having an additional SstI site in the middle of its genome. Clone BH10 contains a viral insert of 9.0 Kb, a size consistent with the complete HTLV-III genome. Clones BH8 and BH5 contain inserts of 5.5 Kb and 3.5 Kb, respectively, and together they overlap completely with BH10, except for a few restric-

tion enzyme sites polymorphisms in BH5. Therefore, BH10 and BH8 plus BH5 represent two variants of HTLV-III.

EXAMPLE 1

In order to demonstrate the presence of these two variants in the original cell line, nick-translated inserts of lambda BH10 was hybridized to a Southern blot of H9/HTLV-III genomic cDNA digested with several restriction enzymes (Figure 3). Both forms could be detected using the enzyme SstI generating the expected 3 bands of 9.0 Kb, 5.5 Kb and 3.5 Kb XbaI which does not cut the provirus generating a high molecular weight genome representing polyclonal integration of the provirus and a band of approximately 10 Kb which could be interpreted as representing unintegrated viral DNA since a band of identical size was also present in the undigested first preparation (Figure 1). This was confirmed by Southern blot hybridization of undigested cellular DNA. The existence of unintegrated viral DNA thus explains the presence of a 4 Kb and 4.5 Kb EcoRI fragment seen in both first and total cellular DNA preparations (Figure 1 and Figure 3). BglII and HindIII both cut the LTR and generated the expected internal bands. Several faint bands in the HindIII digest, in addition to the internal bands, represent either defective proviruses or another variant form with differences in the HindIII restriction pattern. The lack of HTLV-III sequences in the uninfected H9 cell line and the uninfected parental line HT as well as in normal human thymus demonstrated the exogenous nature of HTLV-III and showed that the virus does not contain any human cellular sequences. The same results were obtained using nick-translated inserts from lambda BH5 and lambda BH8.

EXAMPLE 2

The availability of the cloned HTLV-III genome allowed sequence homology between HTLV-III, HTLV-I and

HTLV-II to be evaluated. Replicate Southern blots of restriction enzyme digested clones representing the complete genomes of HTLV-I, HTLV-Ib, HTLV-II and GALV as a control were hybridized to full length HTLV-III probe under relaxed conditions. The filters were then washed (λ BH10) under conditions of low, medium and high stringencies in order to estimate the extent of homology between HTLV-III and these viruses (Figure 4). This experiment showed that there is specific homology between HTLV-III, HTLV-I, HTLV-Ib and HTLV-II but not with HTLV-III and GALV. As demonstrated, hybridization of HTLV-III to other members of the HTLV family could be detected at the values of -42°C and -28°C, conditions under which no hybridization to GALV was seen (Figure 4, panels C and D). Of note, the restriction fragments showing greatest homology correspond to the gag/pol region of HTLV-I and to an apparently non-overlapping portion of the pol region of HTLV-II (assuming that the genomic arrangement is similar to that of HTLV-I). Further analysis revealed that it is the 5' half of the gag and the gap between gag and pol which has the greatest homology in HTLV-I. Finally, in HTLV-Ib (a variant of HTLV-I) hybridization to the px region could be seen (1.4 Kb Pst fragment) as well as to the corresponding px fragment in HTLV-I (2.1 Kb Pst/Sst) on the original autoradiogram.

EXAMPLE 3

Figure 2 shows the restriction map of three clones designated λ BH10, λ BH5 and λ BH8 which correspond in size to the three SstI fragments shown in Figure 1. Comparison of these maps suggests that λ BH5 plus λ BH8 constitute one HTLV-III genome, and λ BH10 another. The two viral forms differ in only three out of 21 mapped enzyme sites, including the internal SstI site. As expected, the phage inserts of λBH5 and λ BH8 hybridize under high stringency conditions to λBH10 but not to each other as analyzed by Southern blot hybridization and

electron microscopic heteroduplex analysis. To show the presence of LTR sequences in the clones and to determine their orientation, a cDNA clone (C15) was used as a probe and contained U3 and R sequences. This clone strongly hybridized to the 0.5 Kb BglII fragment of λ BH10 and λBH8, orienting this side 3', and faintly hybridized to the 0.7 Kb SstI/PstI fragment of λ BH5 and λBH10, orienting this side 5', and demonstrated that SstI cuts the LTR of HTLV-III in the R region.

EXAMPLE 4

The presence of two variant forms of HTLV-III in the original cell line was demonstrated by hybridizing the radiolabelled insert of λBH10 to a Southern blot of H9/HTLV-III genomic DNA digested with several restriction enzymes (Figure 3). Both forms were detected using the enzyme SstI which generated the expected 3 bands of 9Kb, 5.5 Kb and 3.5 Kb length. Both of these forms are also present as integrated proviruses because they have been cloned along with their flanking cellular sequences from a genomic library of H9/HTLV-III. Furthermore, XbaI, which does not cut the provirus, generated a high molecular weight smear representing polyclonal integration of the provirus and a band of approximately 10 Kb, representing unintegrated viral DNA. This came 10 Kb band was also detected in undigested H9/HTLV-III DNA, again indicating unintegrated viral DNA. The presence of unintegrated viral DNA also explains the 4 Kb and 4.5 Kb EcoRI fragment seen in both the Hirt and total cellular DNA preparations (Figures 1, 3). Bgl II and Hind III both cut the LTR and generate the expected internal bands. Several faint bands, in addition to the internal bands using Hind III, represent either defective proviruses or another variant form present in low copy number. The lack of HTLV-III sequences in the DNA of the uninfected H9 cell line and the uninfected parental cell line HT as well as in normal human thymus clearly demonstrates the

exogenous nature of HTLV-III and shows that the virus does not contain human cellular sequences. The same results were obtained using λ BH5 and λ BH8 as probe inserts.

WHAT IS CLAIMED IS:

1. Recombinant clone BH10 characterized by containing the complete HTLV-III genome.

2. Recombinant clone BH8 characterized by containing a 5.5 Kb viral insert from HTLV-III virus.

3. Recombinant clone BH5 characterized by containing a 3.5 Kb viral insert from HTLV-III.

4. A process for the production of recombinant molecular clones of HTLV-III consisting essentially of cleaving unintegrated viral DNA from HTLV-III cells with a restriction enzyme to obtain a provirus, hybridizing radiolabeled cDNA to said provirus, and digesting said virus in a suitable plasmid.

5. A process for the molecular cloning and expression of cDNA sequence of HTLV-III consisting essentially of

isolating total cellular mRNA from H9/HTLV-III cells;

forming double-stranded cDNA from said mRNA and inserting said double-stranded cDNA into a phage lambda to form a recombinant DNA molecule;

hybridizing said recombinant DNA molecule with a radiolabelled probe;

removing cDNA from said molecules and inserting said cDNA into a suitable plasmid; and

transfecting said plasmids into a suitable host cell capable of expressing HTLV-III DNA sequences.

6. A process of Claim 5 wherein said plasmid is λ BH10.

7. A process of Claim 5 wherein said plasmid is λ BH8.

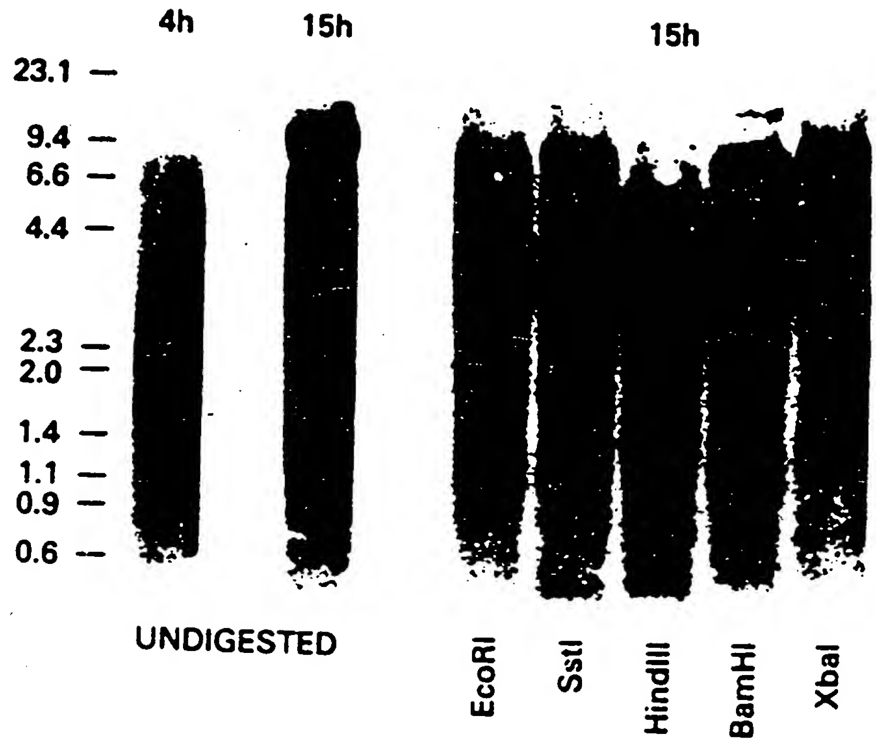
8. A process of Claim 5 wherein said plasmid is λ BH5.

9. A process of Claim 5 wherein said cDNA sequence corresponds to 9.0 Kb sequence.

10. A process of Claim 5 wherein said cDNA sequence corresponds to a 5.5 Kb sequence.

11. A process of Claim 5 wherein said cDNA sequence coresponds to a 3.5 Kb sequence.

FIG.1


4h
15h
15h
23.1
9.4
6.6
4.4
2.3
2.0
1.4
1.1
0.9
0.6
UNDIGESTED
EcoRI
SstI
HindIII
BamHI
XbaI

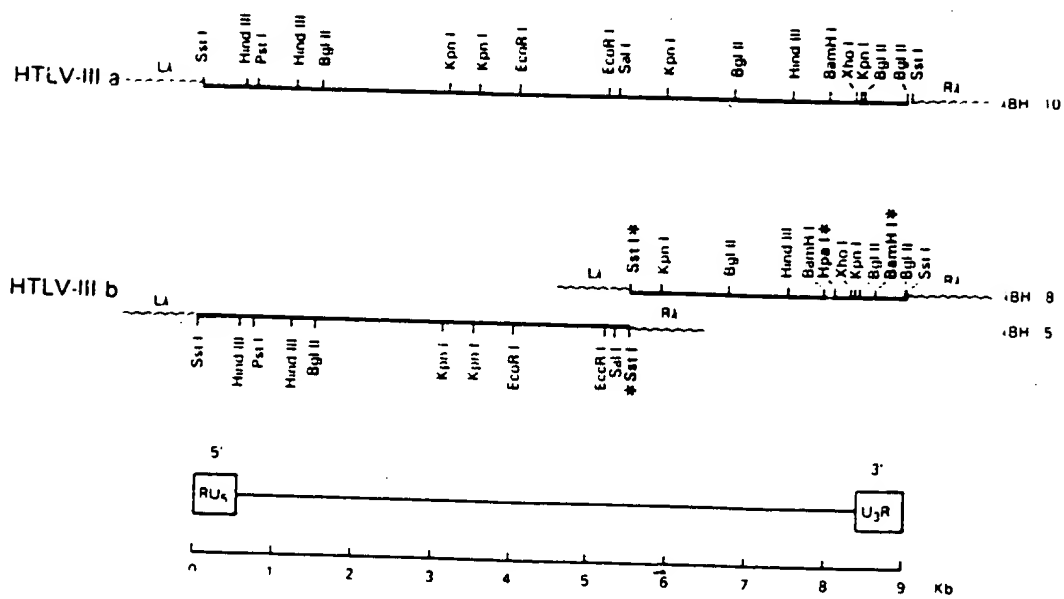
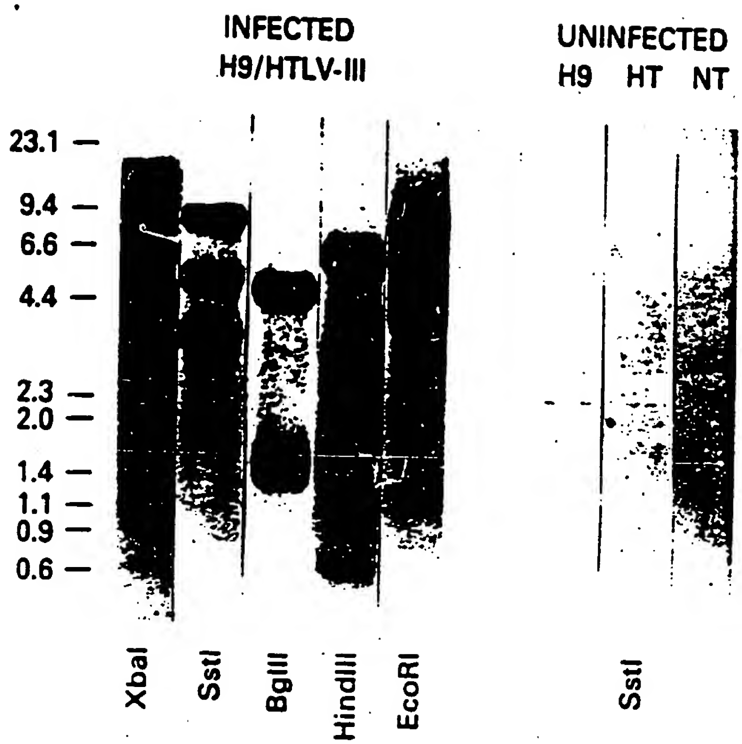

HTLV-III a
HTLV-III b
5'
3'
RU5
U3R
Kb

FIG.2

FIG.3


INFECTED
H9/HTLV-III
UNINFECTED
H9 HT NT
23.1
9.4
6.6
4.4
2.3
2.0
1.4
1.1
0.9
0.6
XbaI
SstI
BglII
HindIII
EcoRI
SstI

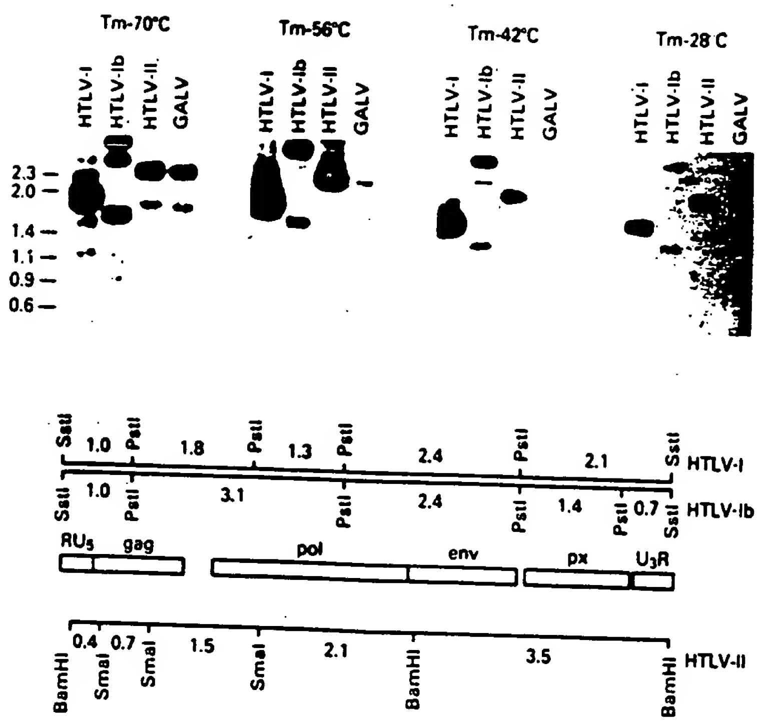

Tm-70°C
Tm-56°C
Tm-42°C
Tm-28°C
HTLV-I
HTLV-Ib
HTLV-II
GALV
2.3
2.0
1.4
1.1
0.9
0.6
SstI 1.0 PstI 1.8 PstI 1.3 PstI 2.4 PstI 2.1 SstI HTLV-I
SstI 1.0 PstI 3.1 PstI 2.4 PstI 1.4 PstI 0.7 SstI HTLV-Ib
RU5 gag pol env px U3R
BamHI 0.4 SmaI 0.7 SmaI 1.5 SmaI 2.1 BamHI 3.5 BamHI HTLV-II

FIG.4

INTERNATIONAL SEARCH REPORT

International Application No PCT/US85/01601

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL 4 C12N 15/00, C12N1/00

II. FIELDS SEARCHED

MAIL ROOM SEP 21 1993 PAT. & TRADEMARK OFFICE

Minimum Documentation Searched [4]	
Classification System	Classification Symbols
U.S.	435/172.3, 317; 935/12, 29, 32, 79

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

COMPUTER DATA BASES: CHEM. ABSTRACTS FILES 309, 310, 311 320; BIOSIS FILES 5,55 MEDLINE FILES 153 154; EMBASE FILE 72, 73, 172, 173

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	N, Seiki et al, Proc. Natl, Acad. Sci. USA vol 80 pp 3618-3622 June 1983	1-11
Y	N, Gallo et al, Science vol 220 pp 866-868 May 20, 1983	1-11
Y	N, Gelmann et al, Science vol 220 pp 862-865 May 20, 1983	1-11
Y	N, Barre'-Sinoussi et al, Science vol 220 pp 868-871 May 20, 1983	1-11
Y	US,A, 4401756 Published Aug. 30, 1983	1-11

* Special categories of cited documents: [15]

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
10/18/85	06 NOV 1985
International Searching Authority [1]	Signature of Authorized Officer [10]
ISA/US	Alvin E. Tanenholtz Alvin E. Tanenholtz